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Specialized expression of simple O-glycans along the rat kidney nephron

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Glycosyltransferases can exhibit tissue-specific expression. By histochemistry glycosyltransferases and their products can be localized to specific cell types in organs of complex cellular composition. We have applied the lectin Amaranthin, having a nominal specificity for Galβ1,3GalNAcR and Neu5Ac2,3Galβ1,3GalNAcα-R, and a monoclonal antibody raised against Galβ1,3GalNAcαR to examine the distribution of these simple O-glycans in adult rat kidney. The monoclonal antibody stained ascending thin limbs of Henle, distal convoluted tubules, and collecting ducts of cortex and outer medulla. Remarkably, the ascending thick limb of Henle, located between ascending thin limb and distal convoluted tubules, was unreactive. However, Amaranthin staining was detectable in ascending thick limbs of Henle, in addition to the structures positive with the monoclonal antibody. In kidney extracts, two bands of approximately 160 kDa and >210 kDa were reactive with both Amaranthin and the monoclonal antibody. One band at ~200 kDa, and a smear at ~100 kDa, were reactive only with Amaranthin. Our data show that in rat kidney simple O-linked glycans are expressed in a highly specialized manner along the renal tubule and can be detected only on a few glycoproteins. This may reflect a cell-type-specific expression of the corresponding glycosyltransferases.

Key words: amaranthin/histochemistry/glycoprotein/kidney/O-glycans

Introduction

Cloning of glycosyltransferases and studies on their tissue distribution have provided a quantum leap in our understanding of the molecular and cell biology of glycosylation (for reviews, see Paulson and Colley, 1989; Kleene and Berger, 1993; van den Eijnden and Joziasse, 1993; Natsuka and Lowe, 1994; Lo *et al.*, 1998). Thus, the analysis of the expression of various glycosyltransferases by Northern blot analysis and specific enzyme assays has revealed their differential tissue expression (Paulson *et al.*, 1989; Kitagawa and Paulson, 1994; Gersten *et al.*, 1995; Johnston *et al.*, 1995; Saito *et al.*, 1995; Smith *et al.*, 1996; Tsuji 1996; Yoshida *et al.*, 1996; Miyoshi *et al.*, 1997). Based on this, the current view is that cell-type-specific glycosylation sequences in general reflect the expression of the respective glycosyltransferases (Paulson *et al.*, 1989; Kitagawa and Paulson, 1994). Retrospectively, these data provided a rationale for the explanation of histochemical studies demonstrating specific expression patterns of glycoconjugates in tissues and cells (for reviews, see Rambourg, 1971; Roth 1978, 1996; Schrevel *et al.*, 1981; Spicer *et al.*, 1981; Reid and Park, 1990). The combined analysis of glycosyltransferase, and its glycosylation product distribution by immuno- and lectin-histochemistry, has substantiated the view that specific glycosylation sequences reflect the expression of particular glycosyltransferases (Roth *et al.*, 1985; Roth, 1997). Moreover, in situ histochemical studies provide a distinct advantage over Northern blot analyses and specific enzyme assays. Glycosyltransferases and their product of action can be localized to specific cell types in situ in organs and tissues which commonly exhibit a complex cellular composition. With the application of lectins and monoclonal antibodies, histochemistry therefore represents an important tool in studies of cell-type-specific glycosylation and may provide a clue about possible functions.

Rat kidney is an organ of complex architecture and different specialized cell types can be distinguished along the renal tubule (Kaissling and Dorup, 1995). Like any other mammalian cell types (Rambourg *et al.*, 1966), kidney epithelia express a glycocalyx rich in carbohydrates and have been shown to exhibit a remarkable degree of variability in their composition, both in the developing and adult organ (LeHir and Dubach, 1982; LeHir *et al.*, 1982; Roth *et al.*, 1983, 1987; Kerjaschki *et al.*, 1984; Kunz *et al.*, 1984; Holthöfer and Virtanen, 1987; Laitinen *et al.*, 1987; Lackie and Roth, 1991). For instance, polymers of α2,8-linked sialic acids present in oligosaccharide side chains of the neural cell adhesion molecule NCAM in rat and human kidney, exhibited not only a developmentally related expression but also a differentiation-dependent pattern of cellular expression (Lackie *et al.*, 1990; Roth *et al.*, 1987). A striking example of heterogeneity in cell-type-specific glycoconjugate expression, depending on the location of a particular cell type in rat kidney, was reported by Brown *et al.*, (1985). In a study on intercalated cells, they observed that this cell type in collecting ducts of cortex, and outer stripe of outer medulla expressed, terminal N-acetylgalactosamine residues, as detected by *Helix pomatia* and *Dolichus biflorus* lectin binding. In contrast, intercalated cells present in the inner stripe of outer medulla and inner medulla were unreactive.

In the course of our studies on the cell type-specific expression of glycoconjugates, we have performed an investigation

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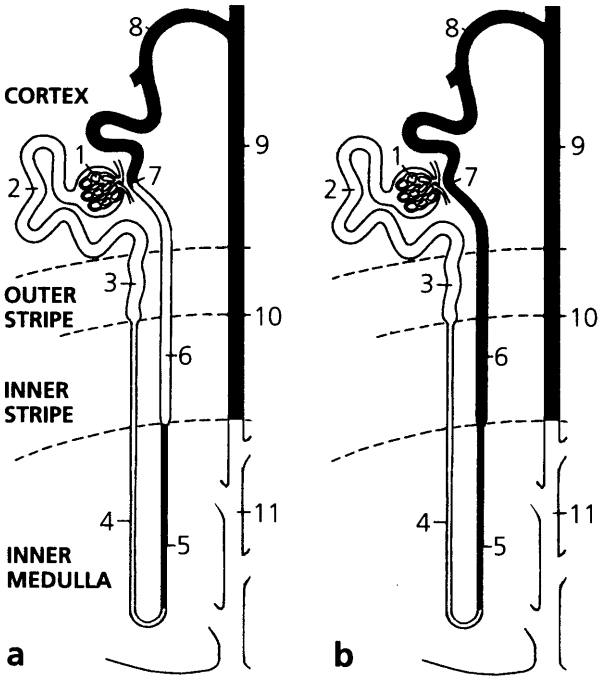

Fig. 1. Schematic presentation of the renal tubule of rat kidney. The distribution of the Galβ1,3GalNAcα- glycan, indicated by black filling of the corresponding part of the renal tubule, is shown in (**a**) and that of the Neu5Acα2,3Galβ1,3GalNAcα- glycan in (**b**). 1, Glomerulum; 2, proximal convoluted tubule; 3, proximal straight tubule; 4, descending thin limb; 5, ascending thin limb; 6, ascending thick limb (distal straight tubule); 7, macula densa; 8, distal convoluted tubule; 9, cortical collecting duct; 10, outer medullary collecting duct; 11, inner medullary collecting duct.

on the expression of simple O-glycosidically linked oligosaccharides in rat kidney. For this we have applied the lectin Amaranthin and a monoclonal antibody raised against synthetic Galβ1,3GalNAcα–R for histochemistry and blot analysis. The protein-bound disaccharide Galβ1,3GalNAcα represents the so-called Thomsen-Friedenreich glycotope, proposed to be a general carcinoma-associated antigen in human beings (Brockhausen, 1993; Springer, 1995; Hanisch and Baldus, 1997). Amaranthin has been reported to bind avidly and equally well to both Galβ1,3GalNAcα–R and Neu5Acα2,3Galβ1,3GalNAcα–R (Rinderle *et al.*, 1989, 1990). Their in situ distinction can be achieved by pretreatment of the tissue sections with galactose oxidase, to selectively oxidize terminal galactose residues, followed by the Schiff reagent (Sata *et al.*, 1990). The present paper reveals the binding sites for Amaranthin and the monoclonal antibody and provides evidence that simple O-glycans in adult rat kidney exhibit a specialized cellular expression and seem to be present only on some glycoproteins.

Results

In the result description, we are following the standard nomenclature for structures of the kidney by Kriz and Bankir (1988). With regard to the specificity of the lectin Amaranthin, we "silently" assume that its binding to rat kidney sections is due to the presence of structures to which it has been shown to bind

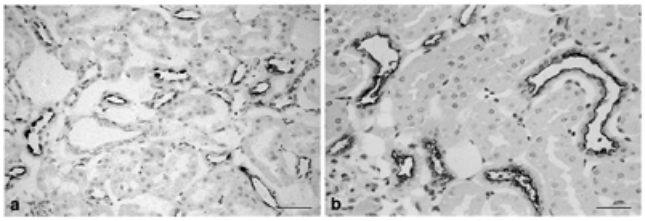

Fig. 2. Rat kidney cortex, paraffin section, silver intensified lectin-gold and immunogold labeling. The silver-intensified gold labeling appears in black in this and all following figures. Both the monoclonal antibody (**a**) and Amaranthin (**b**) stain distal convoluted tubules. The proximal tubules are unreactive. Scale bars, 33 μm.

most avidly (Rinderle *et al.*, 1989, 1990), the Galβ1,3GalNAcα -sequence and the Neu5Acα2,3Galβ1,3GalNAcα-sequence. We notice that lectin-histochemistry does not formally prove the presence of these sequences but for simplicity always refer to these structures.

Binding of the monoclonal antibody, raised against the synthetic Galβ1,3GalNAcα-disaccharide, occurred only to specific parts of the renal tubule. In general, immunolabeling was weak and restricted to the apical (luminal) surface of the positive epithelia. Amaranthin binding principally matched the pattern of monoclonal antibody binding, albeit one additional segment of the renal tubule was reactive. Although lectin labeling was more intense, it was also restricted to the apical cell surface. The labeling patterns in kidney are schematically presented in Figure 1.

The pretreatment of tissue sections with the galactose oxidase/Schiff reaction had no significant influence on the intensity of Amaranthin labeling. Therefore, the lectin labeling can be interpreted to preferentially indicate presence of the Neu5Acα2,3Galβ1,3GalNAcα- sequence. Under the various control conditions for the specificity of antibody and lectin labeling (for details, see *Materials and methods*), no staining was observed (not shown).

Amaranthin and the monoclonal antibody bind to specific parts of the renal nephron

The glomeruli, as well as proximal convoluted and straight tubules and descending thin limbs of Henle of the renal tubule, were unreactive with both the monoclonal antibody and Amaranthin (Figures 2, 3). The positively reacting profiles present in the inner medulla corresponded to ascending thin limbs (Figures 2a,b, 3a,b). Their distinction from descending thin limbs and capillaries became unequivocally possible by electron microscopic labeling, since the two types of thin limbs differ significantly in their ultrastructural morphology (Kaissling and Dorup, 1995). As shown in Figure 3c, Amaranthin labeling occurred in the epithelia of ascending thin limbs and was undetectable in descending thin limbs (Figure 3d). The endothelial cells in the inner medulla (Figure 3c,d) and all other kidney regions were unreactive with Amaranthin and the monoclonal antibody. At the border region of the inner to the outer medulla, the transition of ascending thin limbs into ascending thick limbs (or distal straight tubules) occurs. As can be seen in Figure 4a,b, immunolabeling with the monoclonal antibody ceased at this transition and the ascending thick limbs were unreactive until the macula densa (Figure 4d). In contrast,

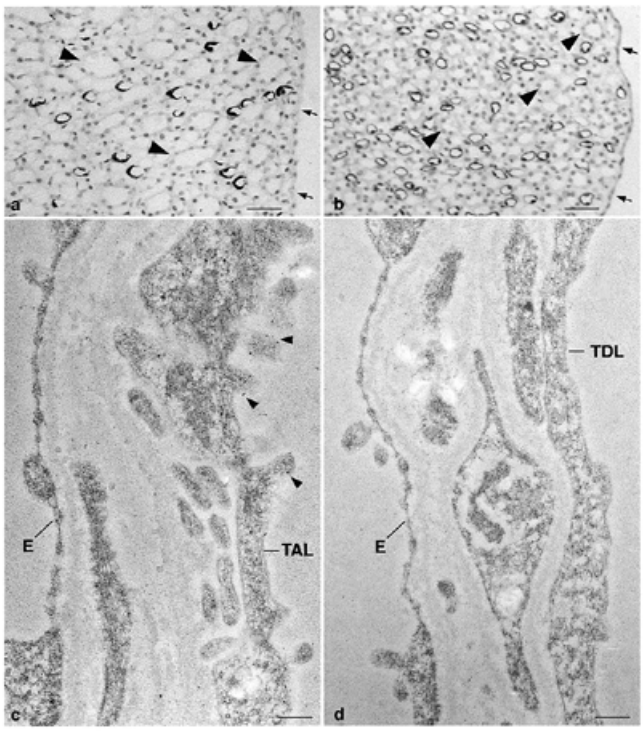

Fig. 3. Details from inner medulla. In paraffin sections, one type of profile is labeled with the monoclonal antibody (**a**) and Amaranthin (**b**). However, inner medullary collecting ducts (arrowheads in **a**, **b**) are clearly unlabeled. In ultrathin sections of Lowicryl K4M embedded inner medulla, the ascending thin limb (TAL) of Henle is positive with Amaranthin (arrowheads in **c** point to gold particle labeling), whereas the descending thin limb (TDL in **d**) of Henle and the endothelia (E in **c** and **d**) of capillaries show no lectin labeling. The epithelium of ascending thin limbs characteristically shows many interdigitated cell processes (**c**), whereas descending thin limbs are composed of flat, non-interdigitating cells (**d**). Arrows in (**a**) and (**b**) point to the transitional epithelium covering the papillary surface. Scale bars, 50 μm (**a**, **b**); 0.25 μm (**c**, **d**).

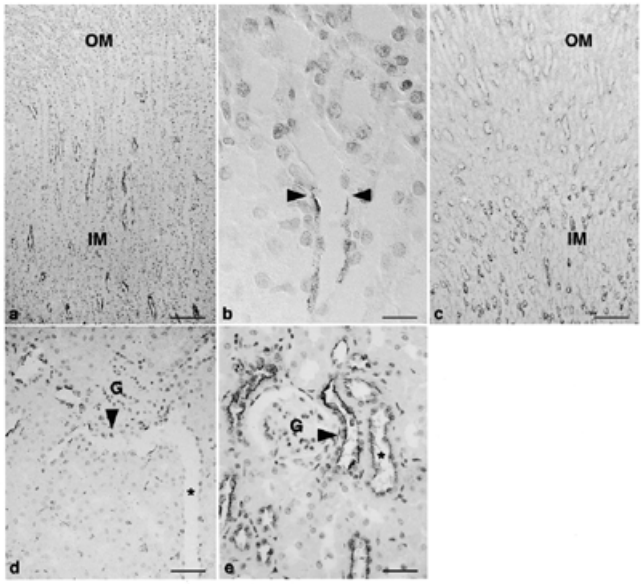

Fig. 4. Transition between inner medulla (IM) and outer medulla (OM) of rat kidney. Labeling with the monoclonal antibody is apparent in the inner medulla but undetectable in the outer medulla (**a**). At higher magnification, the abrupt loss of immunostaining from the ascending thin limb into the ascending thick limb (arrowheads in **b**) and its presence in distal convoluted tubules (**d**) can be seen. The asterisk in (**d**) indicates an unlabeled ascending thick limb of Henle. In contrast, Amaranthin labeling is detectable not only in ascending thin limbs and distal convoluted tubules, but also in ascending thick limbs (**c**, asterisk in **e**). G, Glomerulum; arrowheads in (**d**) and (**e**) point to the macula densa. Scale bars: 85 μm (**a**), 18 μm (**b**), 55 μm (**c**), 33 μm (**d**), 65 μm (**e**).

Amaranthin labeling continued to be positive along the entire ascending thick limbs (Figure 4c,e). Distal convoluted tubules were labeled by both the monoclonal antibody and Amaranthin (Figure 2a,b). The connecting tubule, representing the short transition from distal convoluted tubules into cortical collecting ducts located in the medullary rays, reacted positively with both reagents (Figure 5a,c). Collecting ducts in the medullary rays and in the outer medulla are composed of two cell types, the intercalated cells and the principal cells. Intercalated cells, as compared to principal cells, exhibited stronger immunolabeling with the monoclonal antibody (Figure 5b). Amaranthin labeling was equally intense for both cell types (Figure 5c). Collecting ducts located in the inner medulla were unreactive for both Amaranthin and the monoclonal antibody (Figure 4a, b).

Immuno- and lectin blot analysis

Homogenates from whole kidney and dissected cortex, as well as inner medulla, were analyzed. Unequivocal separation of outer medulla from cortex and inner medulla could not be achieved under our conditions. Only a few reactive bands were observed in blots from the tissue extracts resolved by gradient SDS–PAGE. In kidney cortex, two bands with an apparent molecular mass of ~160 kDa and >210 kDa were reactive with the monoclonal antibody and Amaranthin (Figure 6). In extracts from the cortex and inner medulla, only the 160 kDa band was reactive with both reagents. One band at ~200 kDa and a smear at around 100 kDa were reactive only with Amaranthin (Figure 6). They were not due to the presence of blood, since the results were the same with kidneys which had been washed free from blood by vascular perfusion (not shown).

Discussion

The present study indicates that in rat kidney simple O-linked glycans are expressed in a highly specialized manner along the renal tubule and can be detected only on a few glycoproteins. As demonstrated by Amaranthin and monoclonal antibody labeling, certain parts of the renal tubule, such as the ascending thin limbs of Henle, distal convoluted tubules and collecting ducts of cortex and outer medulla, were reactive with both reagents. Remarkably, a single part of the renal tubule, namely, the ascending thick limb of Henle, only exhibited labeling with Amaranthin. Based on the nominal specificity of Amaranthin and the monoclonal antibody, this labeling pattern can be assumed to represent the distribution of both the Galβ1,3GalNAcα- and Neu5Acα2,3Galβ1,3GalNAcα-sequences. However, this awaits proof by additional structural analysis.

Glycosyltransferases involved in the synthesis of the O-glycosidically linked glycans have been extensively studied (Sadler, 1984; Brockhausen, 1995; Clausen and Bennett, 1996;

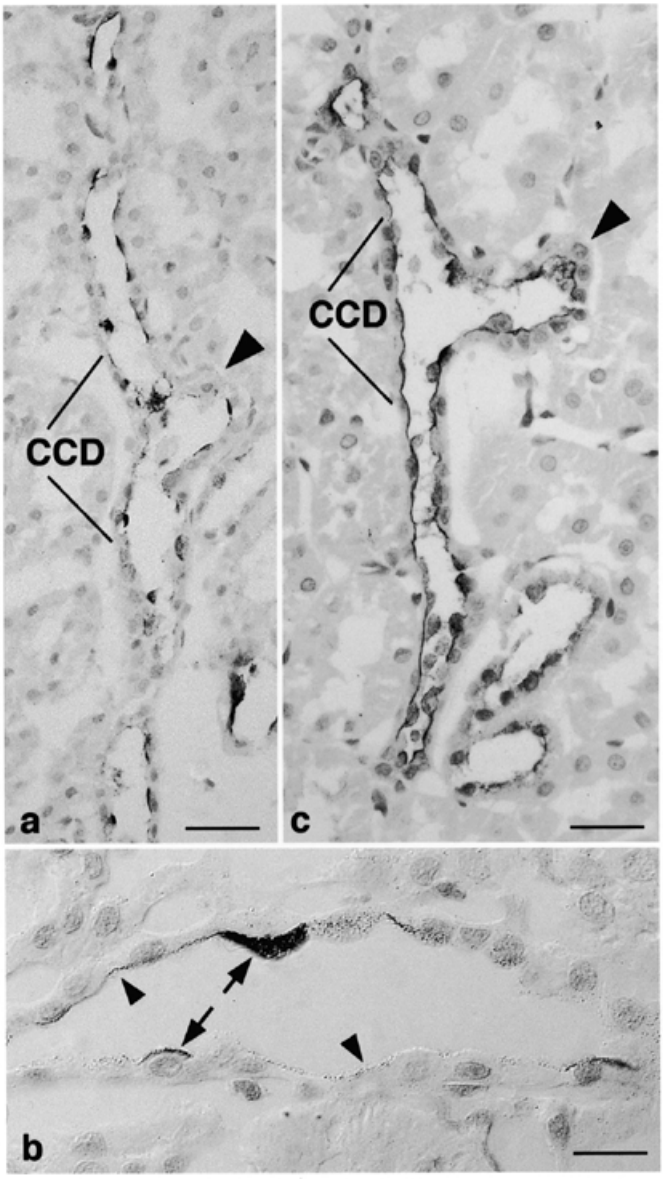

Fig. 5. Cortical collecting ducts (CCD) exhibit labeling with the monoclonal antibody (**a**, **b**) and Amaranthin (**c**). Immunostaining is more intense in the intercalated cells (arrows in **b**) than in the principal cells (arrowheads in **b**). Arrowheads in (**a**, **c**) point to connecting tubules. Scale bars: 25 μm (**a**, **c**), 10 μm (**b**).

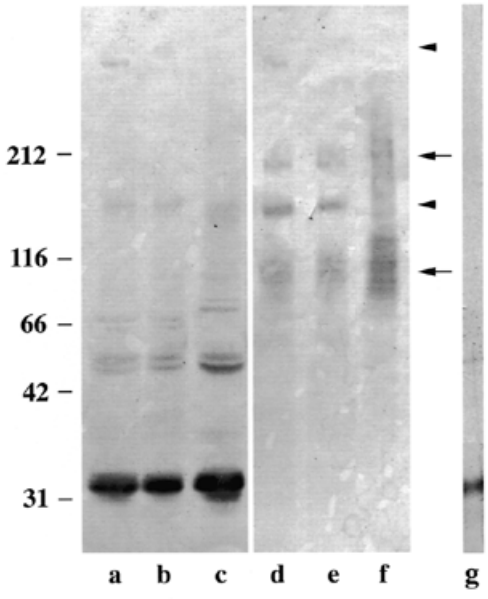

Fig. 6. Blot analysis of whole kidney (lanes a and d), cortex (lanes b, e) and inner medulla (lanes c, f) homogenates by monoclonal antibody (lanes a–c) and Amaranthin (lanes d–f). The monoclonal antibody binds to a >210 kDa and a 160 kDa glycoprotein, as indicated by the position of the arrowheads. Additional Amaranthin binding is indicated by the arrows. Note that the >210 kDa and the 160 kDa glycoproteins are reactive with both reagents. The additional bands seen in lanes a–c, are due to crossreactivity of the goat anti-mouse IgG antibodies with endogenous rat immunoglobulins as presented in lane g which shows tissue homogenate only incubated with alkaline phosphatase conjugated goat anti-mouse IgG.

Marth, 1996). All current evidence indicates the existence of a family of UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases exhibiting distinct substrate specificities (Hagen *et al.*, 1993; Homa *et al.*, 1993; White *et al.*, 1995; Bennett *et al.*, 1996; Clausen and Bennett, 1996, 1996; Zara *et al.*, 1996; Wandall *et al.*, 1997). There is evidence, based on Northern blot analysis, that they exhibit variable cell and tissue expression (Clausen and Bennett, 1996; Marth, 1996). Our present data of selected binding of Amaranthin and a monoclonal antibody do not exclude the presence of UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases in other parts of the renal tubule. Actually, binding of blood group A reactive lectins, such as *Helix pomatia* and *Dolichus biflorus* lectin, to glomeruli and proximal convoluted and straight tubules has been reported (Roth *et al.*, 1983; Brown *et al.*, 1985). The core 1 β3-galactosyltransferase is the second acting glycosyltransferase in the O-glycan biosynthetic pathway and synthesizes the Gal β1,3GalNAcα–R (Brockhausen 1993), a carcinoma-associated glycotope in human beings (Brockhausen 1993; Springer, 1995; Hanisch and Baldus, 1997). As demonstrated by the monoclonal antibody labeling pattern, core 1 β3-galactosyltransferase must be at least present in ascending thin (and ascending thick, see below) limbs, distal convoluted tubules as well as cortical and outer medullary collecting ducts. This indicates that in the epithelia of these parts of the renal tubule, the O-glycans of some glycoproteins are terminated at the core 1 structure. From the results of our blotting analyses, a 160 kDa and a >210 kDa glycoprotein most probably carry such terminated core 1 structures.

The action of the O-glycan 3-sialyltransferase results in another termination reaction yielding the trisaccharide Neu5Acα2,3Galβ1,3GalNAcα-R, which may be detected with Amaranthin and distinguished from Gal β1,3GalNAc-R in combination with the galactose oxidase/Schiff reaction (Sata *et al.*, 1990). From the galactose oxidase/Schiff resistant Amaranthin labeling and the blot analysis, it can be concluded that a 200 kDa glycoprotein and glycoprotein(s) at around 100 kDa bear such an O-glycan structure in ascending thick limbs. However, both the terminated O-glycan core 1 structures and their sialylated forms seem to coexist on a 160 kDa and a

>210 kDa glycoprotein in additional segments of the renal tubule (see Figure 1). Rat kidney is an organ rich in sialic acids (Kerjaschki *et al.*, 1984; Schulte *et al.*, 1984; Roth *et al.*, 1987; Roth, 1996). In this regard, the restricted regional expression of galactose oxidase-resistant Amaranthin labeling, indicative of sialylated O-glycan core 1 structures, is remarkable. There is also evidence for the restricted expression of Amaranthin binding in fetal rat kidney (Sata *et al.*, 1990). Other studies have revealed a much broader distribution of sialylated N-glycans. With the use of the *Sambucus nigra* agglutinin (Shibuya *et al.*, 1987), α2,6-linked sialic acid residues could be detected in the glomerula and along the renal tubule of rat kidney (Taatjes *et al.*, 1988). These results were confirmed and extended by Kaneko *et al.*, (1995) using antibodies to Galβ1,4GlcNAcα2,6-sialyltransferase and *Sambucus nigra* agglutinin. Recently, Burger *et al.*, (1998) reported the distribution of immunoreactivity for Galβ1,4GlcNAcα2,3-sialyltransferase in rat kidney but no glycosyltransferase product localization was reported. In their studies, Kaneko *et al.*, (1995) noted that in several cell types the intensity of immunostaining for Galβ1,4GlcNAcα2,6-sialyltransferase did not always correlate with stainability by *Sambucus nigra* agglutinin. It remains to be demonstrated if this observation is due to the known competition of different sialyltransferases for a common acceptor substrate, differences in the branch specificity of sialyltransferases, or different enzyme expression levels. The new combination of laser-assisted single cell picking from tissue sections, with subsequent RT-PCR analysis, is currently employed in our laboratory to help answer some of these open questions.

Materials and methods

Reagents

Digoxigenin labeled Amaranthin and galactose oxidase (GO) were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). A mouse IgM monoclonal antibody (T-001) raised against the anomer of synthetic Thomsen-Friedenreich (Galβ1,3GalNAc) glycotope was purchased from BioCarb Chemicals (Lund, Sweden), polyclonal sheep anti-digoxigenin antibodies (IgG fraction) from Boehringer Mannheim GmbH (Mannheim, Germany) and affinity-purified goat anti-mouse IgG from Jackson ImmunoResearch Labs., Inc. (West Grove, PA). Particles of colloidal gold, with a diameter of 8 nm, were prepared with the citrate-tannic acid method (Slot and Geuze, 1985) and used to label anti-digoxigenin and affinity-purified goat anti-mouse IgG antibodies.

Tissue processing

Rat kidneys were fixed with 3% paraformaldehyde and 0.1% glutaraldehyde in Hanks' balanced salt solution by vascular perfusion (15 min at 37°C), followed by immersion in the same fixative for up to 2 h. Free aldehyde groups were quenched with 50 mM NH_4Cl in PBS. Slices of whole kidney were embedded in paraffin according to standard protocols. Sections (5 μm) were prepared and mounted on Superfrost Plus glass slides. Pieces form inner medulla were embedded at progressively lowered temperature (down to –42°C) in Lowicryl K4M according to the PLT protocol (Roth, 1989). Ultrathin sections were prepared and mounted on parlodion/carbon coated nickel grids.

Lectin-gold and immunogold techniques

For light microscopy, paraffin sections were deparaffinized in two changes of xylene, rehydrated through a series of graded ethanol, and finally brought to PBS. For lectin labeling, sections were incubated with 10 μg/ml Amaranthin-digoxigenin conjugate in PBS containing 1% BSA for 45 min, rinsed twice for 5 min each in PBS, and incubated with anti-digoxigenin antibody-gold complexes for 45 min. The anti-digoxigenin antibody-gold complexes were diluted to an absorbance of 0.05 at 525 nm. Afterwards, sections were rinsed, fixed in 2% glutaraldehyde in PBS for 20 min, rinsed in PBS, then thoroughly in distilled water and air-dried. Silver intensification of gold particles was performed using silver acetate. Sections were counterstained with nuclear fast red. For immunolabeling, sections were incubated with the monoclonal antibody T-001 (20-fold diluted in PBS containing 1% BSA, 0.05% Triton X-100, and 0.05% Tween 20) for 2 h or overnight at 4°C (50-fold diluted), rinsed twice for 5 min each in PBS, and incubated with goat anti-mouse IgG antibody–gold complexes (absorbance of 0.2 at 525 nm) for 45 min. Following rinses with PBS, sections were fixed with 2% glutaraldehyde in PBS for 20 min, and subjected to photochemical silver amplification, as mentioned above.

For electron microscopy, Lowicryl K4M ultrathin sections, mounted on grids, were floated on a droplet of PBS containing 1% BSA and 0.05% Tween 20 for 10 min and transferred to droplets of Amaranthin-digoxigenin conjugate (10 μg/ml in PBS containing 1% BSA and 0.05% Tween 20) for 45 min, rinsed twice for 5 min each in PBS, and incubated with anti-digoxigenin antibody-gold complexes for 45 min. The anti-digoxigenin antibody-gold complexes were diluted to an absorbance of 0.05 at 525 nm. After rinses with buffer and distilled water, grids with the attached sections were air-dried and contrasted with uranyl and lead acetate.

Cytochemical controls

Prior to incubation of tissue sections, Amaranthin-digoxigenin conjugates, as well as monoclonal antibody T-001, were incubated for 20–30 min with asialoglycophorin (10, 20, 50, and 100 μg/ml). Furthermore, sections were incubated only with anti-digoxigenin IgG–gold complexes (absorbance of 0.05 at 525 nm) or with goat anti-mouse IgG antibody–gold complexes (absorbance of 0.2 at 525 nm) followed by photochemical silver amplification.

In conjunction with Amaranthin histochemistry, sections were pretreated with galactose oxidase (50 U/ml, pH 7.0, for 4 h at 37°C, or 5 U/ml overnight at 4°C) followed by Schiff reagent (0.5%) for 15 min at room temperature (Sata *et al.*, 1990). This procedure reveals the presence of Neu5-Acα2,3Galβ1,3GalNAcα-R.

Lectin and immunoblot analysis

Slices (~1 mm thick) from the entire kidney or dissected pieces, containing either cortex or inner medulla of kidney, were frozen in liquid nitrogen and stored at –70°C until use. Tissue pieces were homogenized on ice in PBS containing 1% Triton X-100, 1 mM phenyl methyl sulfonyl fluoride (PMSF), and 1% aprotinin and kept for 30 min on ice before centrifuga-

tion (14,000 × *g* for 10 min). The supernatant was denatured by boiling in Laemmli buffer for 5 min. The samples were electrophoretically resolved in 5–17.5% gradient SDS–polyacrylamide gels and transferred to nitrocellulose essentially according to the method of Towbin *et al.*, (1979) but using a semi-dry blotting apparatus.

Amaranthin blotting was performed as previously published (Zuber *et al.*, 1998). Briefly, nitrocellulose sheets were blocked for 1 h with TBS (0.1 M Tris–HCl, pH 7.4, 0.15 M NaCl) containing 1% BSA and 0.05% Tween 20. Thereafter, the strips were incubated with digoxigenin-conjugated Amaranthin (1 μg/ml) for 1 h and lectin binding to glycoproteins on the nitrocellulose was detected using alkaline phosphatase–conjugated sheep anti-digoxigenin Fab_2 fragments (diluted 1:10,000) for 45 min, followed by the nitroblue tetrazolium/X-phosphate reaction according to the manufacturer′s instructions.

For immunoblotting, nitrocellulose sheets containing the transferred proteins were blocked for 1 h with PBS (0.1 M phosphate buffer, pH 7.4, 0.15 M NaCl) containing 1% defatted milk powder and 0.05% Tween 20, incubated with the monoclonal antibody T-001 (diluted 1:200) for 48 h at 4°C, washed, and incubated with alkaline phosphatase–conjugated goat anti-mouse IgG antibodies for 45 min, followed by the nitroblue tetrazolium/X-phosphate reaction.

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